In this respect, the cellular localization of this peptide and its regulation in various iron states are of major importance in the study of hepcidin function. Although Northern blot analysis of human and mouse hepcidin mRNA levels in various organs revealed that hepcidin is predominantly expressed in liver (Krause et al. (2000) FEBS Lett 489, 147-150; Park et al. (2001) J Biol Chem 276, 7806-7810; Nicolas et al. (2002) Proc Natl Acad Sci USA 99, 4596-4601), no data exists on the cellular localization of this peptide.

SUMMARY OF THE INVENTION

The present invention concerns hepcidin regulation of iron uptake by mammalian cells and the use of hepcidin and/or hepcidin specific antibodies in the diagnosis of diseases involving disturbances of iron metabolism. The diagnostic detection kits of the present invention can be particularly useful in screening the overall population of either humans or animals and identifying those subjects who have these diseases.

One aspect of the invention is a method for diagnosing a disease condition characterized by non-physiological levels of hepcidin, comprising obtaining a tissue or fluid sample from a subject; contacting the sample with an antibody or fragment thereof that specifically binds to a polypeptide from the mid-portion (amino acids 20 to 50) or C-terminus ~~of hepcidin~~ (amino acids 65 to 84) of hepcidin of SEQ ID NO: 2, and quantifying the hepcidin level using an assay based on binding of the antibody and the polypeptide; wherein the non-physiological level of hepcidin is indicative of the disease condition. In one aspect of the present invention, sensitive diagnostic methods and kits were established enabling the detection of prohepcidin in human plasma. The invention opens a broad range of therapeutic perspectives, where a hepcidin antibody and diagnostic methods and kits can be used for the determination of hepcidin as a parameter for the progress of the diseases mentioned above during and after therapy.

shown by dashed lines (from Hunter et al., 20). The antisera EG (1 and 2)-Hep N are raised against hepcidin precursor aa 28-47 (SEQ ID NO: 3), antiserum EG(1)-HepC is raised against aa 70-84 (SEQ ID NO: 4) as denoted by the antibody symbols.

FIG. 2 illustrates as follows: (A): RT-PCR analysis of human liver (lanes 2 and 3) and HepG2 cells (lanes 4 and 5) showing gene expression of hepcidin. A bp DNA ladder is indicated (lanes 1 and 7). Lane 6 show a negative control. (B-D): Western blot analyses of hepcidin in extracts of guinea pig (lanes 1) and human liver (lanes 2) as well as in HepG2 cells (lanes 3), human serum (lanes 4), and guinea pig skeletal muscle (lanes 5, control) with antibodies EG(1)-HepN (B), EG(2)-HepN (C) and EG(1)-HepC (D). Note the immunoreactive bands at 10 and 20 kDa obtained with all antibodies recognizing different epitopes in the hepcidin precursor. (Molecular mass markers used: phosphorylase B, 105 kDa; glutamic dehydrogenase, 53 kDa; carbonic anhydrase, 34 kDa; myoglobin-blue, 23 kDa; myoglobin-red, 17 kDa; lysozyme, 13 kDa; aprotinin, 7 kDa; insulin, 3 kDa.)

FIG. 3 illustrates detection of hepcidin in HepG2 cells by immunofluorescence microscopy using the antibodies EG(1)-HepN (A), EG(2)-HepN (B), and EG(1)-HepC (C) (Scale bar 8 μm).

FIG. 4 illustrates the cellular localization of hepcidin in guinea pig (A-F) and human (G-I) liver. The paraffin sections immunostained with the region-specific antibodies EG(1)-HepN (A, D, G), EG(2)-HepN (B, E, H) and EG(1)-HepC (C, F, I) show a distinct immunoreactivity at the basolateral membrane domain of hepatocytes (arrows). (Magnification: A-C, X 180; D-I, X 540).

FIG. 5 illustrates immunohistochemical sections of guinea pig liver (A, antibody EG(1)-HepN; B, antibody EG(2)-HepN: C, antibody EG(1)-HepC showing the clear zonation of hepcidin within the hepatic lobules with decreasing immunoreactivity from periportal zones (stars) towards the central veins (arrowheads). Note that no

immunoreactivity is found in hepatocytes around the central veins. (The arrow in B indicates a portal triad.) (A-C, X 180).

FIG. 6 illustrates ELISA results for circulating human prohepcidin. A representative standard curve with concentrations of hepcidin-(28-47) (SEQ ID NO: 3) in ng/ml and the extinction of the ELISA solution at 450 nm wavelength are shown. Note the high resolving power in the range of 4 to 400 ng/ml hepcidin-(28-47).

FIG. 7 illustrates box-plot of values of venous serum prohepcidin concentrations in 26 healthy volunteers (control), 40 patients with chronic renal insufficiency, 19 patients with chronic renal insufficiency and renal anemia, and 35 patients with hereditary hemochromatosis. The line within the box indicates the median, the circle indicates the mean. The lower and upper edge of the box indicates the 1st and 3rd quartile, the whiskers the minimum and maximum values. The dashed line marks the mean level of the control group for circulating immunoreactive prohepcidin (106.16 ng/ml).

FIG. 8 illustrates a correlation between prohepcidin and iron (A), ferritin (B) and transferrin saturation (C) in samples of treated and non-treated HH patients. Note that no remarkable correlation was found in our samples.

FIG. 9 illustrates the complete nucleotide (SEQ ID NO: 1) and amino acid sequences (SEQ ID NO: 2) of one form of hepcidin reproduced from GenBank database accession nos. NM021175 and AAH20612, respectively.

FIG. 10 illustrates as follows: (A) RT-PCR analysis of human (lane 2), mouse (lane 3), and rat (lane 4) kidney showing gene expression of hepcidin. A bp DNA ladder is indicated (lanes 1 and 5). (B, C) Western blot analyses of hepcidin in extracts of human (lanes 1), rat (lanes 2), and mouse (lanes 3) kidney, as well as in human urine (lanes 4) with antibodies EG(2)-HepN (B), and EG(1)-HepC (C). Note the immunoreactive bands at 9.5 kDa obtained with

from New England BioLab (Beverly, Mass.), Pharmacia (Piscataway, N.J.) and Invitrogen, respectively. A hepcidin protein can also be tagged with an epitope and subsequently purified by using a specific antibody directed to such epitope. One such epitope ("FLAG®") is commercially available from Kodak (New Haven, Conn.).

Other fragments and derivatives of the sequences of hepcidin proteins/peptides which would be expected to retain protein activity in whole or in part (e.g., binding to a TfR2 receptor, binding to a hepcidin specific antibody, etc.) and are useful for screening or other immunological methodologies may also be easily made by those skilled in the art given the disclosures herein. Such modifications are encompassed by the present invention.

A hepcidin protein or fragment thereof should be immunoreactive whether it results from the expression of the entire gene sequence, a portion of the gene sequence or from two or more gene sequences which are ligated to direct the production of chimeric proteins. This reactivity may be demonstrated by standard immunological techniques, such as radioimmunoprecipitation, radioimmune competition, or immunoblots.

GENERATION OF ANTIBODIES WHICH DEFINE A HEPCIDIN PROTEIN OR FRAGMENT THEREOF

Various procedures known in the art may be used for the production of antibodies to the mid-portion (amino acids 20 to 50) or C-terminus of epitopes (amino acids 65 to 84) of a hepcidin protein of SEQ ID NO: 2. The hepcidin specific antibodies bind those epitopes and no other known sequences. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments and an Fab expression library. For the production of antibodies, various host animals may be immunized by injection with a particular hepcidin protein, or a synthetic hepcidin protein, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response,

suspected of being an agonist or antagonist of hepcidin with hepcidin and transferrin receptor under conditions such that hepcidin is capable of binding to the transferrin receptor; measuring the amount of hepcidin bound to a transferrin receptor; and determining the effect of the substance by comparing the amount of hepcidin bound to a transferrin receptor with an amount determined for a control.

The invention also relates to a method of identifying agonists or antagonists of hepcidin comprising reacting a substance suspected of being an agonist or antagonist of hepcidin with a cell which produces hepcidin, measuring the amount of hepcidin expressed by the cell, and determining the effect of the substance by comparing the amount of expression of hepcidin with an amount determined for a control. The invention further relates to a method for identifying an agonist or antagonist of hepcidin-mediated iron uptake comprising: incubating a cell expressing hepcidin on its surface and a substance suspected of being an agonist or antagonist of hepcidin in the presence of iron and in the absence of transferrin, measuring the amount of iron uptake into the cell, and identifying an agonist or antagonist of hepcidin-mediated iron uptake by comparing the amount of iron uptake in the cell with the amount of iron uptake in a cell from a control incubation in the absence of the substance.

In some embodiments of the invention, hepcidin peptides are provided for therapeutic use in subjects having symptoms of a primary iron overload disease or syndrome, such as hemochromatosis, or other iron overload condition caused by secondary causes, such as repeated transfusions. A hepcidin peptide can be full-length hepcidin or some fragment of hepcidin. Preferably, a hepcidin peptide comprises the amino acid residues 28 to 47 or 70 to 80 of a hepcidin (SEQ ID NO: 2). The predicted amino acid sequence and genomic and cDNA sequences of hepcidin were provided in (Krause et al., (2000) FEBS

Lett. 480, 147-150; Pigeon et al., (2001) J. Biol. Chem. 276, 7811-7819), hereby incorporated by reference in their entirety. A hepcidin protein or fragment thereof may be administered with beta-2-microglobulin, such as in the form of a complex. In some embodiments, a hepcidin protein greater than about 20 amino acids is administered in a complex with beta-2-microglobulin.

In some embodiments of the invention, agonists or antagonists of a hepcidin protein or a transferrin receptor are provided. Agonists of a hepcidin polypeptide, and/or antagonists of a transferrin receptor, are useful for example, in the treatment of primary or secondary iron overload diseases or syndromes, while antagonists of a hepcidin polypeptide, or agonists of the transferrin receptor are useful, for example, in the treatment of iron deficiency conditions, such as anemias. In other embodiments, mutant hepcidin proteins/peptides are provided which function as antagonists of the wild-type hepcidin protein. Antagonists or agonists can also be antibodies, directed against a transferrin receptor, or the mid-portion (amino acids 20 to 50) or C-terminal region (amino acids 65 to 84) of a hepcidin protein (SEQ ID NO: 2). In some embodiments of the invention, hepcidin polypeptides can serve as antagonists of a transferrin receptor. In further embodiments of the invention, peptidomimetics can be designed using techniques well known in the art as antagonists or agonists of a hepcidin protein and/or a transferrin receptor.

Ligands for a transferrin receptor, whether antagonists or agonists, can be screened using the techniques described herein for the ability to bind to a transferrin receptor. Additionally, competition for hepcidin binding to a transferrin receptor can be done using techniques well known in the art. Ligands, or more generally, binding partners for a hepcidin protein can be screened, for example, for the ability to inhibit the complexing of a